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Gastrulation Initiation in *Caenorhabditis elegans* Requires the Function of *gad-1*, Which Encodes a Protein with WD Repeats

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Gastrulation in *Caenorhabditis elegans* is normally initiated by inward migration of the two gut precursor (E) cells at the 26-cell stage. A strong loss-of-function, temperature-sensitive, embryonic lethal mutation in the maternally required gene *gad-1* (gastrulation defective) prevents gastrulation initiation. In embryos from homozygous mutant *gad-1 (ct226)* hermaphrodites reared at 25°C, the E cells divide early with abnormal spindle orientations and fail to migrate into the embryo, and no subsequent gastrulation movements occur. These embryos continue to develop and differentiate the major cell types, but they undergo little morphogenesis. The temperature-sensitive period of the mutant is during early embryogenesis, prior to gastrulation onset. The predicted translation product of the cloned *gad-1* gene includes six β-transducin-related repeats of the WD motif, which has been implicated in protein–protein interactions. The *ct226* mutation alters a conserved residue in one of these repeats. Injection of *gad-1* antisense RNA into wild-type hermaphrodites mimics the mutant phenotype in progeny embryos. We conclude that the *gad-1* gene product is required for initiation of gastrulation in *C. elegans*.

Key Words: WD repeats; endoderm; spindle orientation.

INTRODUCTION

Gastrulation in *Caenorhabditis elegans* is initiated by inward movement of the two daughters of the embryonic founder cell E, which gives rise to all 20 cells in the adult gut and contributes cells to no other tissues (Sulston *et al.*, 1983). Between the 8-cell and 15-cell stages (Fig. 1), the E cell divides once in an anterior–posterior (A/P) direction to produce Ea and Ep on the ventral surface. Ea and Ep contact the vitelline membrane, which lines the interior of the chitinous egg shell surrounding the embryo. Gastrulation initiates when Ea and Ep leave the membrane and begin to migrate, with no evident changes in their shapes or those of the neighboring cells, away from the ventral surface toward the center of the embryo. Ingression of Ea and Ep forms a ventral cleft (Fig. 2A) through which descendants of the other five founder cells (AB, MS, C, D, and P_4) then migrate. The E cells are followed by the four granddaughters of the mesodermal precursor MS from the anterior margin of the cleft and by the germline cell P_4 and the myoblast D from the posterior margin. During migration of these cells, Ea and Ep divide internally (the 2E4 division) in a direction that is primarily left–right (L/R) but also has a dorsal–ventral (D/V) component (Fig. 2B). As additional AB- and C-derived mesodermal precursors migrate inward, the E cells divide further in the A/P direction, and by about the 180-cell stage, they have formed a gut rudiment (Sulston *et al.*, 1983), which can be seen clearly as two rows of large cells in the center of the embryo (Fig. 2C). Shortly thereafter, these cells begin to accumulate autofluorescent, refractile, cytoplasmic rhabditin granules (gut granules), which serve as a convenient marker for gut differentiation (Laufer *et al.*, 1980).

Although *C. elegans* gastrulation is relatively simple, involving the ingression of only 53 cells, little is known about its mechanism (see Bucher and Seydoux, 1994, for review). Some clues to the requirements for gastrulation initiation and progression have come from embryo manipulation experiments. When Ea and Ep are killed by laser irradiation, they do not migrate into the embryo, and none of the subsequent cell movements of gastrulation occur (Junkersdorf and Schierenberg, 1992). Further experiments indicated that the inward movement of Ea and Ep may be

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cell-autonomous because elimination of P_2 and MS cells by extrusion does not prevent the E-cell migration (Junkersdorf and Schierenberg, 1992). In addition, gastrulation movements seem to depend on integrity of the vitelline membrane, which renders the embryo impermeable to most ions and solutes. Making large perforations in this membrane that cannot quickly reseal will arrest gastrulation, even if it has already initiated (Schierenberg and Junkersdorf, 1992).

A general prerequisite for gastrulation appears to be correct specification of the E-cell fate. E is formed by division of the EMS cell in the third cycle of embryonic cleavage (Fig. 1). Specification of EMS requires maternally supplied *skn-1* product to distinguish EMS from its sister blastomere P_2 (Bowerman *et al.*, 1992, 1993). EMS then requires a polarizing signal from P_2 in order to give rise to two different daughter blastomeres, E and MS (Goldstein, 1992, 1993). Polarization of EMS requires genes of a Wnt-related signaling pathway, including the *mom* genes *mom-1* through *mom-5* (Rocheleau *et al.*, 1997; Thorpe *et al.*, 1997) and *pop-1* (Lin *et al.*, 1995). Subsequently, specification to produce gut granules also appears to depend upon functions of the maternally required *gut* genes (J. Shaw, personal communication) and the embryonically required *end-1* (Zhu *et al.*, 1997).

Several genes that affect gastrulation have been mutationally identified (see Discussion), but control of this process and its relationship to endodermal differentiation are still unclear. To better understand the control of gastrulation, we have begun to identify and characterize additional genes necessary for its initiation. We describe here a new maternal-effect gene, *gad-1* (gastrulation defective). The temperature-sensitive loss-of-function mutant *gad-1 (ct226)* completely fails to initiate gastrulation at nonpermissive temperature and exhibits a temperature-sensitive period (TSP) consistent with an essential role in this process. The *gad-1* gene encodes a novel protein including six WD motifs, similar to those found in β-transducins; these repeats are thought to mediate protein interactions. One of the repeats is altered by the *ct226* mutation, suggesting that GAD-1 may be required to interact with other proteins in controlling gastrulation initiation.

MATERIALS AND METHODS

Strains and Alleles

The Bristol N2 wild-type and all mutant strains derived from it were constructed by standard genetic procedures from stocks in our collection or obtained from the *Caenorhabditis* Genetics Center, University of Minnesota, St. Paul, and cultured as described by Sulston and Hodgkin (1988). The single allele defining *gad-1V, ct226*, was isolated as described below. Strains used for mapping included:

dpy-18(e364)/eT1 III; nDf18/eT1[let-500(s2165)]
dpy-18(e364)/eT1 III; sDf26/eT1 V
dpy-11(e224) unc-42(e270) V
unc-83(e1408) dpy-11(e224) V

Isolation of gad-1(ct226ts) V

The *ct226* mutant was isolated as a viable strain at 16°C following EMS mutagenesis in a screen for temperature-sensitive mutants with reversal of gonad handedness (Wood and Kershaw, 1991). It was backcrossed to N2 five times prior to genetic mapping and phenotypic analysis. At 16°C, the majority of embryos hatch and develop into fertile adults with <10% penetrance of the gonad reversal phenotype (W.B.W., unpublished). Self-progeny embryos from hermaphrodites reared at 20 or 25°C arrest with the embryonic-lethal, gastrulation-defective phenotype described here (see Results).

Phenotypic Analysis by Light Microscopy

Routine observation and scoring of embryonic phenotypes was carried out with a Wild dissecting microscope and a Leica DX-4 compound microscope equipped with Nomarski, polarization, and fluorescence optics. The majority of inviable embryos were scored using the dissecting microscope to observe their stage of arrest at 100× magnification. Embryos that arrested at the lima bean stage with no visible morphogenesis were scored as gastrulation defective. For each experiment, a sample of embryos was viewed under Nomarski and polarization optics, which allows gut granules to be seen as refractile bodies. Gastrulation-defective embryos were defined as embryos containing gut granules that remained posterior and on the embryonic surface at terminal arrest stage (in late embryos that have executed gastrulation, these cells are seen in the embryonic interior). Development of selected embryos was recorded using a multi-focal-plane, time-lapse video microscopy system (4D microscope) modified from a published description (Thomas *et al.*, 1996). It consisted of the above Leica microscope equipped with a Kohu video camera and a Power Macintosh computer including a Scion LG3 8-bit frame grabber NIH Image software was used for image acquisition and control of the camera. A series of through-focus optical sections in steps of 1 μm was recorded every 30 s for 3–4 h while maintaining the embryo at approximately 20 or 25°C.

Immunofluorescence Microscopy

The procedures used have been described in detail by Powell-Coffman *et al* (1996). Gravid *gad-1(ct226)* hermaphrodites grown at 25°C overnight were transferred into a drop of M9 and cut with a scalpel to release the embryos. Two-cell embryos were isolated, treated with a 1:10 dilution of sodium hypochlorite in egg salts, rinsed, and cultured in egg salts at 25°C for 7 h to undergo differentiation before fixation and staining. The monoclonal antibodies used were MH27, which recognizes adherens junctions and outlines the hypodermal cells, and MHC-A, which recognizes body wall muscle fibers (both antibodies kindly provided by R. Waterston).

Germline Transformation Rescue of the Gad-1 Phenotype

Homozygous *gad-1(ct226)* hermaphrodites were microinjected with combinations of cosmids, single cosmids, or subcloned constructs, all at total concentrations of 20–40 ng/μl in a solution that also contained 100 ng/μl DNA carrying *rol-6(su1006)*, a dominant Roller (Rol) marker used to identify transformants (Mello *et al.*, 1991). F1 progeny with the Rol phenotype were picked, five to a

plate, at 16°C. F2 broods that contained Rol animals were used to establish transgenic lines, and these lines were tested for rescue of the *ct226* phenotype by placing L4 hermaphrodites at 25°C. Survival of the F3 progeny indicated rescue.

Generation of ^{32}P-Labeled Probes

All probes were made by random primer extension (Sambrook *et al.*, 1989). The probes used to screen the cDNA library were made from two PCR products that included the 5′ 1 kb of the genomic *gad-1* sequence. The probes used for RNA blot analysis were generated from two subclones of *gad-1* cDNA (pJK22 and pJK26, which together included the entire cDNA), and from *act-1* (pCcA103; Krause *et al.*, 1989). Five hundred nanograms of template was used to produce 800 Ci/mmol α-^{32}P-labeled probe by standard random priming procedures (Sambrook *et al.*, 1989). The probe was run through a Sephadex G-50 column and assayed for ^{32}P incorporation in a scintillation counter.

cDNA Library Screen

^{32}P-labeled probes described in the preceding section were used to screen 6×10^5 plaque-forming units from a *C. elegans* mixed-stage cDNA library (Okkema and Fire, 1994). From the primary screen, 10 positive clones were isolated and plated at 10^4 dilution. Secondary and tertiary lifts were screened with the same probe, and then positives were tested by PCR with primers recognizing *λgt11* and one or more internal sequences. Of the three positives, one (B8a3) was chosen for sequencing. When compared to the gene structure predicted by Genefinder (Sulston *et al.*, 1992), the sequence revealed that all but the first 6 bp of the *gad-1* cDNA were present in the clone. A full-length cDNA was obtained by using custom-designed primers (GIBCO-BRL) to add the first 6 base pairs of the cDNA to the 5′ end. To determine whether *gad-1* was SL1-spliced, DNA was reverse transcribed from RNA made from N2 embryos. A PCR product, amplified by using an SL1 primer and an internal *gad-1* primer, was then sequenced and verified to carry SL1 at its 5′ end. The 5′ 1.1 kb of the *gad-1* cDNA was ligated into the polycloning site of the Bluescript vector to generate pJK22, which was used for generation of antisense RNA.

Synthesis of Antisense RNA

pJK22 (preceding section) was linearized at one end of the cDNA by restriction enzyme digestion. *In vitro* transcription of RNA was performed as detailed in Powell-Coffman *et al* (1996). RNA was extracted with acid phenol and chloroform, precipitated with 1/10 vol of 0.8 M LiCl and 2.5 vol of 100% EtOH and resuspended in diethylpyrocarbonate-treated water. The RNA concentration was determined with a spectrophotometer.

Antisense Injection and Scoring of Progeny

Antisense RNA at concentrations indicated in the text was injected as described above for germline transformation. Injected hermaphrodites were allowed to recover for 12 h at 16°C before scoring progeny. Animals were then transferred singly to new plates every 12 h for 24 h. Eggs laid were counted immediately after the hermaphrodite was transferred to a new plate, and terminal phenotypes were scored 16–20 h later.

RNA Blot Analysis

RNA was prepared as previously described (Schauer and Wood, 1990). Approximately 10 μg of RNA was loaded in each lane for electrophoresis. RNA was then transferred to a nylon membrane, which was hybridized sequentially with an *act-1* and then a *gad-1* probe. Hybridizations and exposures were performed as detailed in Sambrook *et al.* (1989).

RESULTS

Phenotypic Characterization of a gad-1 Mutant

The maternal-effect, temperature-sensitive embryonic lethal mutant *gad-1(ct226)* was found to be defective in the initiation of gastrulation at nonpermissive temperatures. As described in the Introduction, gastrulation normally initiates at the 26-cell stage when Ea and Ep move away from the ventral surface of the embryo and then undergo division internally with a L/R orientation. In embryos produced by a *gad-1* homozygous hermaphrodite, there are three major alterations in this process. (1) Ea and Ep and their daughters have shortened cell cycles, so that Ea and Ep divide prematurely during the time when they would normally be moving inward (Figs. 1 and 2D). (2) Ea and Ep divide in the wrong orientation, A/P rather than L/R (Fig. 2E). (3) Neither Ea and Ep nor their progeny move inward; all E descendants remain on the ventral surface throughout embryogenesis (Fig. 2F). Presumably as a consequence of this behavior, the other blastomeres that normally ingress are prevented from doing so as well, so that no gastrulation movements occur. All five *gad-1* mutant embryos in which cell lineages were followed had a shortened E-cell cycle at 25°C, though there was variability in its length. In wild-type embryos, Ea and Ep typically divide 25 min after the 2MS4 division (see Fig. 1), with Ea always dividing shortly before Ep (Sulston *et al.*, 1983; J.K., unpublished observations). In the *gad-1* mutant embryos, Ea still divided before Ep, but these divisions occurred from 4.5 to 10 min after the 2MS4 division. Differences in the shortening of the E-cell cycle did not appear to be correlated with any differences in terminal phenotype.

Although *gad-1* mutant embryos do not execute the normal cell movements required for correct positioning of most tissues, they nevertheless undergo differentiation of many tissue types. Most of the cell interactions required for determination of early blastomere fates take place before the onset of gastrulation (reviewed in Schnabel and Priess, 1997; Wood and Edgar, 1994). Lineage analysis of *gad-1* mutant embryos up to the 200-cell stage indicated that the cells in all other lineages except E divided at the normal times and in the correct orientations in 5/5 embryos, but failed to execute gastrulation movements. As indicated in Fig. 3, all mutant embryos differentiate hypodermal and muscle cells, although these tissues are positioned incorrectly. Gut differentiation, based on the presence of gut granules, occurs in about 90% of *gad-1* mutant embryos, and differentiated gut cells are always clustered at the

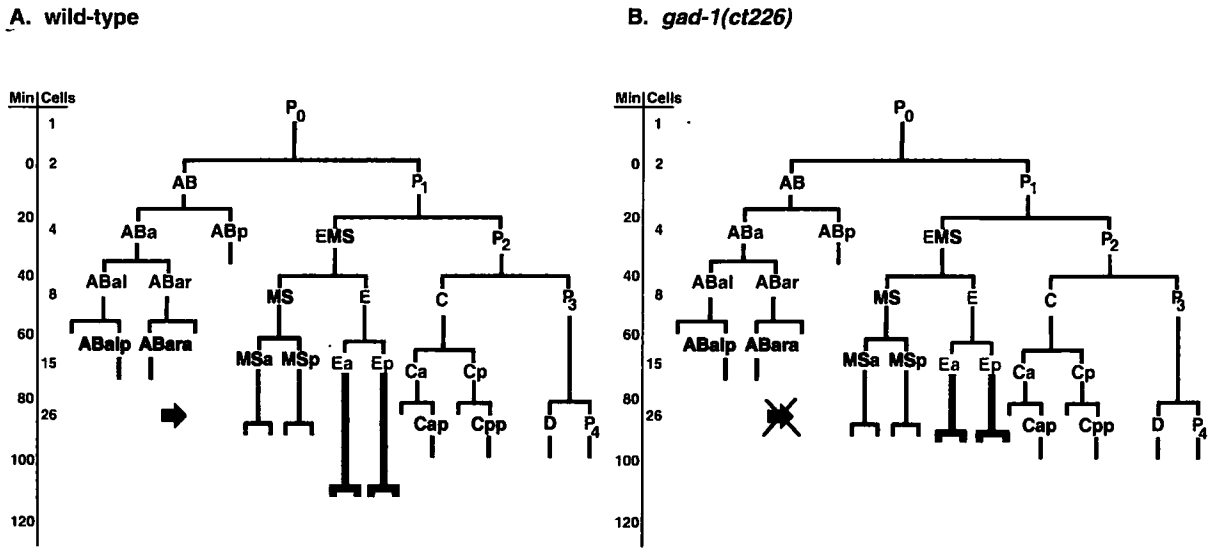

FIG. 1. Early lineage relationships of gastrulating embryonic cells. Founder cells of the major somatic lineages are designated by convention as AB, MS, E, C, D; their progeny are named according to division orientation (e.g., ABa and ABp are anterior and posterior daughters of AB, respectively). The P cells constitute the germline. Horizontal bars indicate blastomere divisions at the times and embryonic stages shown on the vertical axis. Cells whose progeny ingress during gastrulation are shown in color and include endodermal (blue), germline (purple), mesodermal (red), and pharyngeal (green) precursors. Founder cells that give rise to multiple cell types are shown in multiple colors. Blue arrow indicates the time at which Ea and Ep normally begin the inward migration that initiates gastrulation. In *gad-1(ct226)*-mutant embryos (B), Ea and Ep divide prematurely at about this time, and their progeny remain on the ventral surface. *E*-lineage cell-cycle times affected by the *gad-1* mutation are shown by heavy lines.

posterior end of the animal, usually superficially. In occasional morphogenesis-defective animals, gut granules are not superficial and located at the posterior; however, examination of the cell movements in many *gad-1*-mutant embryos indicates that posterior superficial gut granules are always indicative of a failure to gastrulate.

Genetic Characterization of gad-1(ct226)

Three factor mapping of *gad-1(ct226)* placed it approximately 0.02 map units to the left of *dpy-11* near the center of LG V (Fig. 4A). The *ct226* allele is temperature-sensitive and exhibits a strict maternal effect; that is, the mutant oocytes produced by a homozygous hermaphrodite cannot be rescued by a *gad-1(+)* allele from male sperm (Table 1). At 25°C, 100% of the embryos produced by such a hermaphrodite arrest with the gastrulation defect described above. Gene dosage experiments using the deficiency *nDf18*, which uncovers *gad-1*, suggest that *ct226* is a hypomorphic allele: at 16°C *gad-1/nDf18* heterozygotes laid 90% inviable embryos, compared to 31% (see Table 1) laid by *gad-1(ct226)* homozygotes. All embryos from *gad-1/nDf18* heterozygotes are gastrulation defective, while a small percentage of the inviable embryos from *gad-1* homozygotes at both 16 and 20°C arrest later with a partially developed pharynx but a disorganized posterior. In addition to the maternal-effect phenotype, *gad-1(ct226)* results in a nonmaternal viable phenotype of variable gonad positioning, including about 10% of animals with reversed gonad handedness, which we have not further investigated (W.B.W., unpublished).

The temperature-sensitive period (TSP) for *gad-1* function is consistent with a role in gastrulation initiation for maternally synthesized GAD-1 protein. The start of the TSP is from early to mid-oogenesis (Fig. 5); time spent at 25°C by eggs in the hermaphrodite parent between the start of oogenesis and the embryonic 28-cell stage results in a decreased frequency of embryos that undergo gastrulation. The end of the TSP is at the 28-cell stage, indicating that once gastrulation has initiated, *gad-1* function is no longer necessary for its completion. This result suggests that GAD-1 may be critical for the initial inward movement of Ea and Ep but not for the migration of the other cells that normally follow.

gad-1 Encodes a Protein with WD Repeats

Transformation rescue of *gad-1* embryonic lethality (see Materials and Methods) was attempted with cosmid clones from the *dpy-11* region (Fig. 4A), and was successful with cosmid F45G1. As shown in Fig. 4B, the two smallest subclones of F45G1 to give transformation rescue were pJK15, including two predicted genes, and pJK16, which has a deletion in one of the predicted genes; the second com-

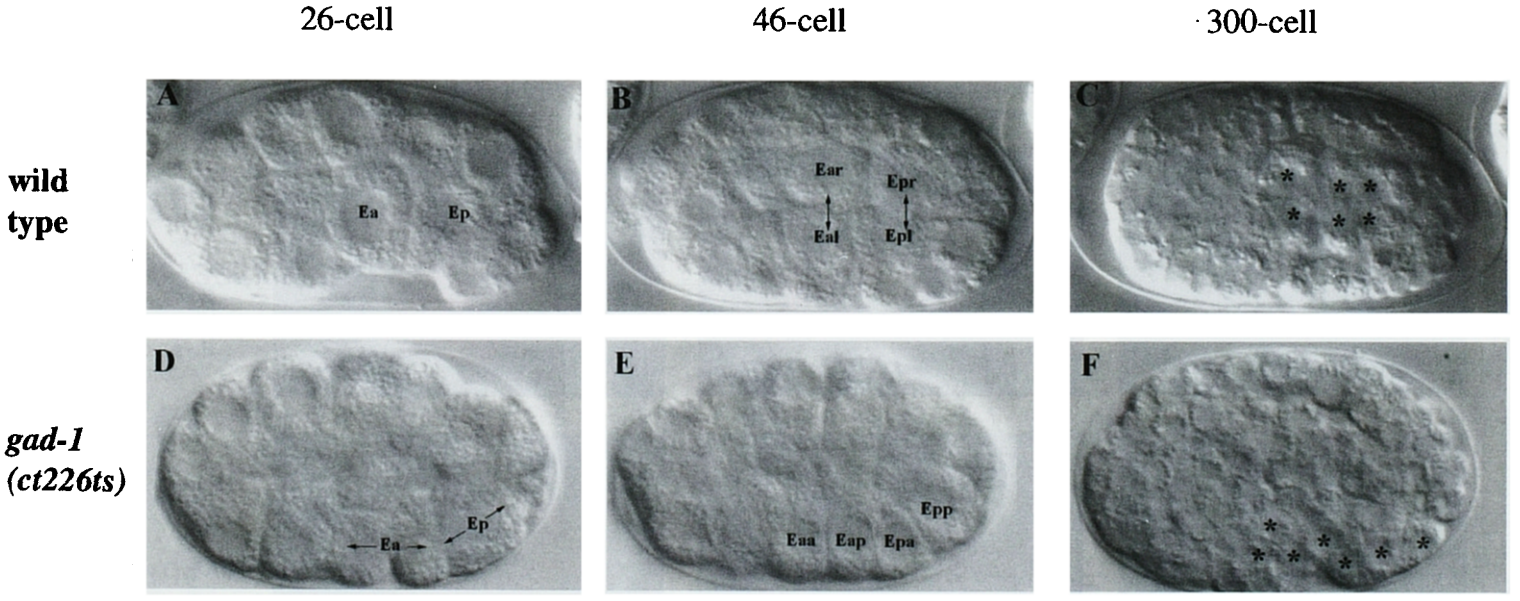

FIG. 2. Effect of the *gad-1(ct226)* mutation on gastrulation. Nomarski images of three stages during gastrulation are shown for a wild-type (top panels) and a typical *gad-1(ct226)* (bottom panels) embryo. All images are anterior-left, dorsal-up. The endodermal precursors (Ea and Ep) are labeled. At the 26-cell stage, Ea and Ep have begun to migrate inward in wild type (A), while in *gad-1*, they divide precociously and in the A/P direction (D). At the 46-cell stage, the 4 E cells are in the middle of the embryo in wild type, after having divided in an L/R–D/V direction (B), while they are still on the ventral surface in *gad-1* (E). By the 300-cell stage, the 8 E cells (asterisks) have formed a gut primordium of two parallel rows in wild type (C), while they are still on the ventral surface in *gad-1* (F). Not all E cells can be seen in this plane.

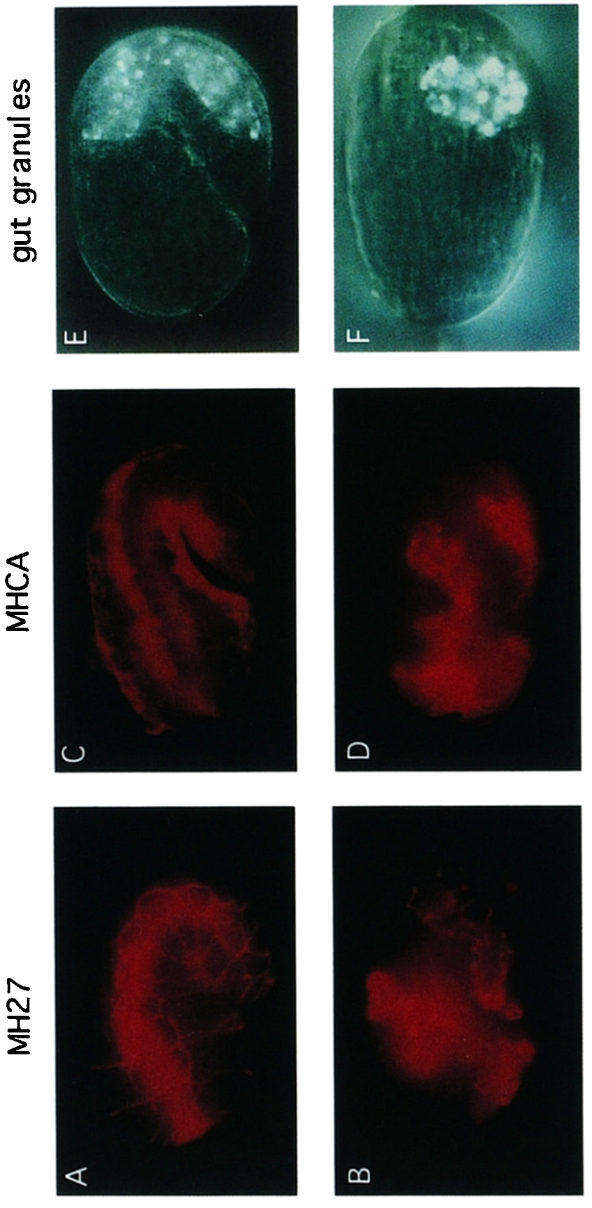

FIG. 3. Differentiation of hypodermal, muscle, and gut cells in *gad-1*-mutant embryos. A and B show embryos stained with MH27, an antibody that recognizes adherens junctions and thus outlines the hypodermal cells. C and D show staining with MHCA, an antibody that recognizes body wall muscle. E and F show embryos viewed with polarized light to visualize the refractile rhabditin granules (gut granules), which appear only in descendants of the E cell. Note that in F the gut granules are clustered at the posterior.

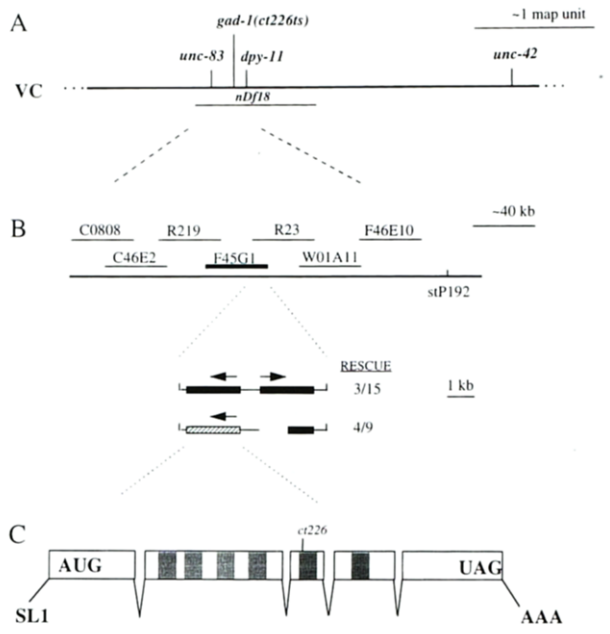

FIG. 4. Genetic mapping and cloning of *gad-1*. (A) The positions of several known genes and the deficiency *nDf18* are shown near the center of LGV. (B) Cosmids between the left endpoint of *nDf18* (C. Malone, personal communication) and the cosmid that rescues *dpy-11* are shown on the physical map. The rescuing cosmid F45G1 is shown in bold. Two subclones generated from this cosmid (pJK15 and pJK16) are shown with an indication of their transformation rescue efficiency (rescuing lines/total transgenic lines obtained). The smallest rescuing subclone was a 4.6-kb fragment in which the promoter sequence had been deleted from the second of two predicted genes. Shaded box indicates the *gad-1* gene. (C) Structure of the *gad-1* cDNA, with position of the *ct226* mutation indicated. Shaded boxes indicate the coding regions for WD repeats. The *gad-1* gene is included in cosmid T05H4 (GenBank Accession No. AF016452), which overlaps F45G1; *gad-1* corresponds to the predicted gene T05H4.14.

plete gene was surmised to be *gad-1*. This identification was confirmed by sequencing of *gad-1(ct226)* mutant genomic DNA, which revealed a single base pair mutation changing a conserved His (see below) to an Asn in the predicted amino acid sequence. Sequencing of a 1.9-kb *gad-1* cDNA and comparison to the genomic sequence showed that the mRNA is SL1-spliced, contains 5 exons, and has an open reading frame predicted to encode a protein of 620 amino acids (Fig. 4C). The position of the *gad-1(ct226)* mutation is shown in the third exon.

The predicted amino acid sequence of GAD-1 does not exhibit striking overall similarity to any protein in the current databases. However, it includes several copies of a motif that has been called the WD repeat for the two C-terminal amino acids in its consensus sequence, Trp and Asp, although some WD repeats have other residues at these positions. To be included in the WD-repeat family, a protein need only have one repeat containing three of the four highly conserved amino acids (W,D,G, and H) and at least one more repeat containing one of these four amino acids (Neer *et al.*, 1994). The predicted GAD-1 protein has 6 WD repeats, which can be aligned to a generic consensus sequence generated from 27 WD-containing proteins (Neer *et al.*, 1994) as shown in Fig. 6. The *gad-1(ct226)* mutation alters the conserved His in the fifth WD repeat. BLAST analysis indicates that the predicted GAD-1 protein is most similar to β subunits of the G proteins known as transducins, in which WD repeats were first identified (Fong *et al.*, 1986). However, the similarities between G_β subunits and GAD-1 are confined to the WD repeats, suggesting that GAD-1 does not act as a β-transducin.

RNA-Mediated Inactivation of gad-1 Phenocopies the Mutant Defects

Because we have not yet isolated a null allele of *gad-1*, we attempted to ascertain the phenotype resulting from lack of maternally derived *gad-1* transcripts by injecting antisense RNA generated from the full-length *gad-1* cDNA. This technique, as applied to *C. elegans*, has been shown to effectively deplete functional mRNAs for a variety of maternally transcribed genes (e.g., Guo and Kramer, 1989; Mello *et al.*, 1996; Powell-Coffman *et al.*, 1996; Rocheleau *et al.*, 1997). The mechanism is not well understood, but has been referred to as RNA-mediated interference or inactivation (RNAi) (Rocheleau *et al.*, 1997). In four experiments with two different concentrations of RNA, 89% of the total progeny embryos from hermaphrodites injected with *gad-1* antisense RNA arrested as balls of cells with highly defective morphogenesis, consistent with a failure to gastrulate (Table 2). Lineage analysis of embryos from antisense-injected hermaphrodites revealed an early defective phenotype indistinguishable from that of *gad-1(ct226)*-mutant embryos. In six of six embryos whose cell lineages were followed, the 2E4 cell division was early (between 4 and 15 min after the 2MS4 division, compared to 25 min in wild type), and the 2E4 division orientation was A/P rather than L/R. Subsequently, none of the E-cell descendants migrated into the embryo. Control injections with sense RNA were not carried out because sense and antisense RNAs have been found to cause similar effects in previous applications of this method (Fire *et al.*, 1991; Guo and Kemphues, 1996).

gad-1 Transcript Accumulation Is Developmentally Regulated

To determine the developmental expression pattern of *gad-1* and to ascertain whether its transcript might be differentially spliced, we probed a blot of RNAs from different developmental stages of wild-type worms and two germline-defective mutants with the *gad-1* cDNA and a sequence from the *C. elegans* actin gene *act-1* as a control

(Fig. 7). A single transcript of the predicted size was detected at eightfold higher abundance in early embryos than in subsequent embryonic and larval stages. Strong expression in adult hermaphrodites was confined to the germline and developing embryos, as shown by the high level of hybridization to RNA from *fem-2(b245)* adult hermaphrodites, which produce oocytes but no embryos (Chin-Sang and Spence, 1996), compared to the background levels of hybridization to RNA from *glp-4(bn2)* adult hermaphrodites, which lack the germline almost entirely (Beanan and Strome, 1992). These findings are consistent with the strict maternal effect of the *gad-1(ct226)* mutation.

DISCUSSION

We have shown that the recessive, maternal-effect, temperature-sensitive *gad-1(ct226)* mutation results in complete lack of gastrulation movements, both those of the two E cells that normally initiate gastrulation and those that normally follow. This mutation also results in premature division of the E cells with abnormal spindle orientation, but does not prevent the E-cell descendants from differentiating as gut cells in most embryos. At the nonpermissive temperature of 25°C, migration and spindle orientation defects are 100% penetrant. At 16°C, these defects are only partially penetrant, but they are enhanced to 100% penetrance in embryos produced by hemizygous *gad-1(ct226)/Df* hermaphrodites as well as in embryos produced by wild-type hermaphrodites injected with antisense mRNA to cause RNAi with *gad-1* function. We conclude that *gad-1(ct226)* is a strong loss-of-function mutation at nonpermissive temperature, and that *gad-1* function is required for gastrulation initiation.

Other Genes Affecting Gastrulation

How gastrulation is controlled remains largely unknown, but recent results from *Drosophila* (Parks and Wieschaus, 1991; Sweeton *et al.*, 1991; Zusman and Wieschaus, 1985), mouse (Chen *et al.*, 1994; Zhou *et al.*, 1993), and zebrafish (Hammerschmidt *et al.*, 1996; Solnica-Krezel *et al.*, 1996) implicate several genes including *goosecoid* (Blum *et al.*, 1992), *brachyury* (Smith *et al.*, 1991; Wilkinson *et al.*, 1990), *twist* and *snail* (Leptin, 1991), and *concertina* (Parks and Wieschaus, 1991). While these genes and their homologues show expression patterns or defective phenotypes, or both, consistent with involvement in gastrulation, none of them appears to be required for gastrulation initiation.

Analysis of other *C. elegans* mutants indicates that the functions of genes involved in EMS specification are necessary for normal gastrulation. In embryos produced by hermaphrodites homozygous for loss-of-function mutations in *skn-1*, EMS adopts a P_2-like fate and no E cell is produced (Bowerman *et al.*, 1992). In embryos produced by hermaphrodites homozygous for any one of the *mom* genes, E adopts an MS-like fate and no E cell is produced (Rocheleau *et al.*, 1997; Thorpe *et al.*, 1997). These mutant embryos fail to gastrulate, as do embryos from *gut*-mutant hermaphrodites (J. Shaw, personal communication). In embryos mutant for the *end-1* gene, in which the E blastomere takes on a C-like fate, gastrulation sometimes initiates but then arrests (Zhu *et al.*, 1997). In embryos from hermaphrodites homozygous for *pop-1* mutations, which cause MS to adopt an E-like fate so that there are two E founder cells, gastrulation occurs in some embryos (Lin *et al.*, 1995, R. Lin, personal communication).

A few additional maternally required genes that may affect gastrulation more specifically have been genetically identified. Their functions are required for gastrulation to occur but not for subsequent E cell differentiation as scored by production of gut granules. Maternal-effect mutations in any of the genes *emb-5*, *emb-13*, *emb-16*, *emb-23*, or *emb-31* cause failure of initiation or subsequent progression through gastrulation. In all these mutants, the 2E4 division of Ea and Ep occurs prematurely, and in some this division is in an A/P rather than L/R direction. The subsequently produced E descendants remain superficial, producing a patch of cells that make gut granules on the posterior surface of the defective embryos (Denich *et al.*, 1984; Nishiwaki *et al.*, 1993). These observations suggest that control of cell-cycle timing and spindle orientation in the early E lineage may also be important for the gastrulation process. The only one of these genes to be molecularly characterized so far, *emb-5*, is predicted to encode a protein similar to the *Saccharomyces cerevisiae* protein SPT6, which is thought to regulate transcription by affecting chromatin assembly (Nishiwaki *et al.*, 1993).

TABLE 1

Effects of Gene Dosage, Temperature, and Maternal Genotype on Viability of *gad-1(ct226)* Mutant Embryos

Hermaphrodite parent	Temperature[a] (°C)	Inviable embryos (%)	Embryos scored
gad-1(ct226)/nDf18, self-fert[b]	16	90	1,186
gad-1(ct226), self-fert	16	31	11,536
gad-1(ct226), self-fert	20	97	5,105
gad-1(ct226), self-fert	25	100	3,082
gad-1(ct226), mated to N2 males[c]	25	100	198

[a] Hermaphrodites reared at the indicated temperature were allowed to lay eggs, which were subsequently scored for hatching as the measure of viability.

[b] *nDf18* is a deficiency that uncovers the *gad-1* locus.

[c] gad-1(ct226) hermaphrodites were reared at 25°C and mated as young adults to N2 males at 25°C. After a day of producing embryos for scoring, the hermaphrodites were shifted to 16°C on individual plates and allowed to produce viable progeny. In all cases male animals were produced at 16°C, indicating that mating had been successful and that the inviable embryos produced at 25°C included *gad-1/* + genotypes.

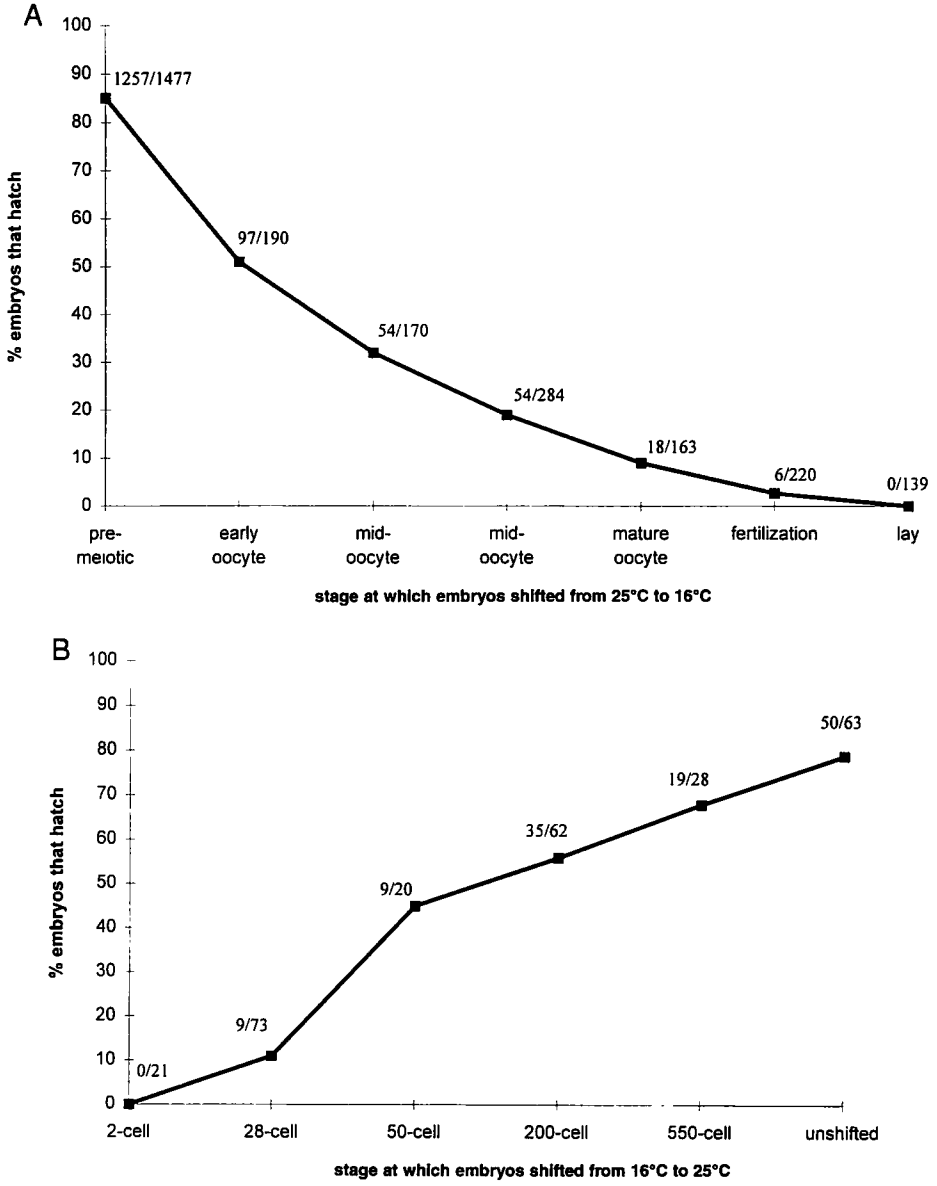

FIG. 5. The temperature-sensitive period (TSP) for *gad-1* function. Percentages of viable (gastrulated) embryos produced after a temperature shift are plotted for shifts of animals or embryos at different stages of oogenesis (A) and embryogenesis (B). The numbers for each point indicate how many embryos match out of the number scored for the shift at that stage. The beginning and end of the TSP are defined as the stages in shift-down and shift-up experiments, respectively, at which 50% of the embryos display the defect (in this case, dead eggs which have failed to gastrulate). (A) To determine the beginning of the TSP, young adult hermaphrodites were incubated at 25°C for 12 h and then shifted to precooled plates at 16°C and allowed to lay eggs. The hermaphrodites were then transferred to new plates at 16°C every 2 h, and progeny were scored 24 h later under the dissecting microscope for viability and execution of gastrulation. A sample of embryos from each time point was also scored under Nomarski and polarization optics for verification. Stages of oogenesis at the time of temperature shift for the embryos scored at each point were estimated from the known timing of germline stages and fertilization at 25°C (Wood *et al.*, 1980). Embryos laid by adults maintained at 25°C and shifted to 16°C at any time after laying invariably failed to gastrulate and did not hatch (not shown). (B) To determine the end of the TSP, 2- to 4-cell embryos isolated from *gad-1* homozygous hermaphrodites reared at 16°C were incubated at 16°C and then shifted to 25°C at the stages shown and scored 24 h later for viability or gastrulation execution as in (A). The stages of embryos at temperature shift were estimated from the known timing of embryonic development at 16°C (Wood *et al.*, 1980).

```
     S
     A  A          A       A A
     C  C          MY Y    C C
     M  M I        CCCC    M M
     F  F L D D    FAAA    F F K
     V  V W G G    VTTT    V VYR
     I  I Y N N    ISSS    I IFN
GHXXXLXXLXFXPXPXXXXLGGGXDXXLXLWD   consensus

HGTQAISALRVEP.PGVRFASGGLDYYVKLFD   1
AESHVINSLAFSP.NGETLVVASGEAIIRLLD   2
GHTATVNCVEFNPLNKNEFTSCSDDGSLRLWN   3
GHRVSPQVCTFSP.DGKWIAAGCDDGSVQAWK   4
*
AHNGSITSIAFSP.DSKRLLSRGFDDTLKMWS   5
YPHODVHRIQWHP.RLNQIIAGLSDGTIHVYY   6
```

FIG. 6. Comparison of the six *gad-1* WD repeats (numbered lines) to the WD motif consensus sequence; the most commonly seen variations are shown above the consensus (Neer *et al.*, 1994). Consensus residues conserved in the *gad-1* repeats are shown in black. Asterisk indicates location of the *ct226* mutation in the fifth repeat.

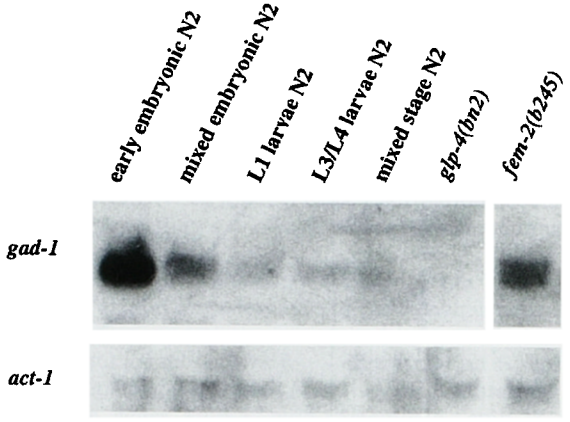

FIG. 7. RNA blot analysis of *gad-1* mRNA accumulation during development. The RNA is most abundant in early embryos and in the germline, as shown by the lanes containing RNA from adult *glp-4* and *fem-2* hermaphrodites. Adult *glp-4* animals essentially lack germline cells; adult *fem-2* animals are effectively females, containing oocytes but no functional sperm or fertilized embryos (see text). Hybridization of the blot to a *C. elegans* actin (*act-1*) probe was used for approximate comparison of total RNA loading in each lane.

We have shown previously that general inhibition of embryonic transcription by injection of antisense RNA to RNA polymerase II also causes premature Ea and Ep division and prevents gastrulation initiation (Powell-Coffman *et al.*, 1996). These findings suggest that maternal-effect genes may regulate one or more embryonically transcribed genes that are essential for gastrulation initiation. Although mutations in the embryonically transcribed genes *end-1* (Zhu *et al.*, 1997) and the ribosomal RNA gene cluster *rrs-1* (Ferguson *et al.*, 1996) cause defects in gastrulation, they do not block its initiation, suggesting that at least one embryonically transcribed gene required for gastrulation initiation remains to be discovered.

TABLE 2

Phenotypes Resulting from RNAi with *gad-1* Antisense mRNA

Experiment	RNA concentration (μg/μl)	% Embryos arrested[a]	Embryos scored
1	0.5	83	2832
2	0.5	92	1723
3	1	95	882
4	1	95	653

[a] All arrested embryos failed to execute gastrulation as assayed with a dissecting microscope.

Fate Specification, Spindle Orientation, and Cell-Cycle Timing

The *gad-1* phenotypic effects add to the evidence from work cited above that there are functional connections among fate specification, spindle orientation, and cell-cycle timing. However, it is also clear that these effects can be separated. In embryos that have an irreversibly disrupted vitelline membrane (Schierenberg and Junkersdorf, 1992), Ea and Ep divide at the normal time but nevertheless do not ingress. Spindle orientations of Ea and Ep were not reported in this study. Conversely, in embryos in which the E-cell cycle was artificially lengthened by brief laser irradiation, the single E cell ingressed at the normal time (Junkersdorf and Schierenberg, 1992). In embryos from *pop-1*-mutant hermaphrodites, there are four E cells on the ventral surface of the embryo rather than two; yet ingression can proceed nevertheless, showing that it is not simply a steric effect of extra E cells that prevents ingression in *gad-1*-mutant embryos (Lin *et al.*, 1995). In most *gad-1* embryos, E descendents express gut-specific gene products, confirming that specification of the E-cell differentiation fate is not sufficient for gastrulation to occur.

gad-1 and Spindle Orientation

gad-1 mutant embryos at nonpermissive temperatures exhibit complete penetrance of the spindle orientation defect in the 2E4 division. The normal centrosomal replication cycle dictates that in successive cleavages of any cell

lineage, the spindle orientation of a given dividing cell will always be orthogonal to the spindle orientation of its parent cell in the previous mitosis, unless the daughter spindle is reoriented before mitosis by some mechanism such as has been demonstrated for the P_1 spindle in the 2-cell *C. elegans* embryo (Hyman, 1989). However, there are many lineages in the *C. elegans* embryo that exhibit successive divisions with the same orientation (generally A/P), suggesting that such reorientations may be frequent. For example, the cleavages of EMS and both of its daughters E and MS are oriented in an A/P direction. In the subsequent round of cleavage, MSa and MSp again divide A/P, and only Ea and Ep, which have moved into the embryo, exhibit the default orthogonal L/R cleavage orientation. In *gad-1*-mutant embryos, the Ea and Ep spindles become reoriented to A/P like those of MSa and MSp. This reorientation could be due to the absence of a signal in the interior that blocks reorientation, or it could reflect misspecification of E to a partially MS-like cell.

Spindle orientation is controlled by several mechanisms (reviewed in Goldstein *et al.*, 1993; White and Strome, 1996), at least one of which involves G-protein signaling. Mutations in the *C. elegans* G_β subunit gene *gpb-1* result in randomization of spindle orientations in early blastomeres (Zwaal *et al.*, 1996). The GAD-1 predicted protein shows sequence similarity to G_β proteins, although only in the WD repeat regions. G_β and G_α subunits complex via several conserved residues in WD repeat regions of the G_β subunits (Gaudet *et al.*, 1996). The finding that the *gad-1(ct226)* mutation alters a conserved His in a WD repeat suggests that these repeats in GAD-1 may serve an essential binding function.

Interestingly, gastrulation in *Drosophila* requires the G_α-encoding gene *concertina (cta)* for ventral furrow formation and posterior midgut invagination (Parks and Wieschaus, 1991). We do not yet know if G_α subunits play a role in *C. elegans* gastrulation; the G_α genes identified so far in *C. elegans* do not have embryonic defects when mutated, but rather affect sensory neurons (Mendel *et al.*, 1995; Zwaal *et al.*, 1997; R. Plasterk, personal communication). However, based on the requirement for at least one embryonically transcribed gene in *C. elegans* gastrulation (Powell-Coffman *et al.*, 1996), it is possible that GAD-1, like Cta in *Drosophila*, has an embryonically transcribed partner. GAD-1 could act as a regulatory protein, binding either directly or indirectly to one of the components of a G-protein signaling pathway which could control downstream genes important for gastrulation, perhaps including the SPT6-like transcription factor encoded by *emb-5*. Screens currently underway for *gad-1(ct226)* suppressors and more gastrulation-defective mutants (unpublished results) should identify additional genes involved in gastrulation initiation and control and provide more information on possible signaling mechanisms.

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